RECOMBINANT FUSOBACTERIUM NECROPHORUM LEUKOTOXIN VACCINE AND PREPARATION THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional application of Application Serial No. 09/841,786 filed April 24, 2001 which was a continuation-in-part application to Application Serial No. 09/558,257, Filed April 25, 2000. The content and teachings of each of these applications is hereby incorporated by reference herein.

SEQUENCE LISTING

A printed Sequence Listing accompanies this application, and also has been submitted with identical contents in the form of a computer-readable ASCII file on a floppy diskette with Application No. 09/558,257, filed April 25, 2000. Use of this previously filed CRF sequence listing is requested.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention is concerned with methods of cloning and expressing the leukotoxin gene from *Fusobacterium necrophorum (F. necrophorum)*, sequencing and characterizing the leukotoxin protein expressed by this gene, truncating the gene into a series of nucleotide sequences, amplifying these sequences, expressing and recovering the polypeptides encoded by the nucleotide sequences, and utilizing the protein and the polypeptides in recombinant vaccines in order to confer effective immunity against infection caused by the production of leukotoxin by *F. necrophorum*. More particularly, it is concerned with production of an inactivated recombinant leukotoxin vaccine generated by amplifying five leukotoxin gene fragments and one upstream region through PCR, digesting the nucleotide sequences encoded by the gene fragments with restriction enzymes, expressing the polypeptide sequences coded by the nucleotide sequences through an expression vector, recovering these proteins as five truncated leukotoxin proteins (or polypeptides), purifying these proteins (or polypeptides) to apparent homogeneity, with or

without inactivation of the truncated and full length proteins, and combining the inactivated recombinant leukotoxins with adjuvants.

Description of the Prior Art

Liver abscesses in feed lot cattle are a serious economic problem, causing condemnation of over 3 million livers and an estimated loss of $15 million annually in the United States. This estimate is based primarily on condemnation of liver and other organs, and does not include economic losses stemming from reduced feed intake, reduced feed efficiencies, decreased carcass dressing percentage and lowered weight gains. A number of studies have confirmed that cattle with abscessed livers gain less (average 4-5%) and have reduced feed efficiencies (average 7%) compared with cattle having healthy livers. The average incidence of abscessed liver in grain-fed cattle approximates 25-30%. To a lesser extent, liver abscesses in sheep and goats are also an economic problem.

F. necrophorum is a gram-negative, rod-shaped, nonsporeforming, nonmotile, strictly anaerobic and pleomorphic organism. Morphologically, the organism varies from short rods to filamentous with pointed and rounded ends. Cell lengths range from coccoid bodies of 0.5-0.7 μm in diameter to filaments over 100 μm. Surface colonies are 1-2 mm in diameter, circular, transparent to opaque, and with some strains producing α or ß hemolysis. The organism ferments glucose, fructose and maltose only weakly with final pH around 5.0-6.3. It ferments lactate to acetate, propionate, and butyrate. Butyrate is the major product from lactate fermentation. Indole is produced from peptone. *F. necrophorum* has been isolated from the normal flora in the oral cavity, gastrointestinal cavity, and genitourinary tract of humans and animals. The organism is also known to survive in the soil.

F. necrophorum is a normal inhabitant of the gastrointestinal tracts of animals and humans. Virulence factors and pathogenic mechanisms that contribute to the transition of this otherwise commensal organism to a pathogen are poorly understood. A leukotoxin, endotoxin, hemolysin, hemagglutinin, and several enzymes such as deoxyribonuclease and proteases have been suggested as possible virulence factors. However, several studies implicate leukotoxin, a protein cytotoxic to ruminant polymorphonuclear cells, as the major virulence factor. The importance of leukotoxin as a virulence factor in *F. necrophorum* infections is indicated by a correlation between toxin production and ability to induce

abscesses in laboratory animals, an inability of nonleukotoxin-producing strains to induce foot abscesses in cattle following intradermal inoculation, and a relationship between antileukotoxin antibody titers and protection against infection in experimental challenge studies.

F. necrophorum is an opportunistic pathogen that is the primary etiologic agent of liver abscesses in ruminant animals. (Scanlan, et al., (1983)Bovine rumenitis-liver abscess complex: a bacteriological review. *Cornell Vet.* 73:288-297; Nagaraja, T. G. et al., (1998) Liver abscesses in feedlot cattle: A review. *J. Anim. Sci.*, 76:287-298; and Tan, et al., (1996) Fusobacterium necrophorum infections: virulence factors, pathogenic mechanism and control measures. *Vet. Res. Comm.*, 20:113-140). The organism has been recognized as an animal and human pathogen since the late 1800s, and is associated as a primary or secondary etiologic agent with numerous necrotic disease conditions in domestic and wild animals. In addition to liver abscesses, the organism is also the primary etiologic agent of foot rot, foot abscesses, calf diphtheria, and is frequently isolated from cases of mastitis, metritis, and necrotic lesions of the oral cavity.

Liver abscesses in cattle are part of a disease complex where the abscessation is secondary to primary foci of infection in the rumen epithelium. The pathogenesis can be summarized as follows: (1) ruminal lesions are induced by acidosis that follows rapid change in diet from high-roughage to high grain, prolonged feeding of high grain diet, or occasionally by foreign body penetration of the rumen epithelium; (2) bacteria present in the rumen invade the epithelium and form focal abscesses in the rumen wall; and (3) bacteria enter the portal circulation, and are carried to the liver where they localize in the parenchyma with subsequent abscess formation.

The ability of *F. necrophorum* to establish in the liver is attributed to the production of a toxin which is a secreted protein of high molecular weight active against leukocytes from ruminants called leukotoxin (or leucocidin). The toxin is a soluble extracellular protein that is cytotoxic to neutrophils, macrophages, hepatocytes, and ruminal cells. The leukotoxin protects against phagocytosis and is believed to aid in the establishment of *F. necrophorum* in the liver by directly impairing the normal defense mechanism and indirectly by the damage caused by cytolytic products released from neutrophils and macrophages to the hepatic cells. Therefore, the leukotoxin elaborated from *F. necrophorum* plays a critical

role in *F. necrophorum* infection of the liver and is believed to be the primary virulence factor in the pathogenesis of liver abscesses (Tan et al., 1996).

Four biotypes (A, B, AB and C) of *F. necrophorum* have been described. (Langworth, (1977) Fusobacterium necrophorum: its characteristics and role as an animal pathogen. *Bacteriol. Rev.* 41:373-390) Biotype A, most frequently isolated from liver abscesses, is more pathogenic than biotype B, which predominates in ruminal wall abscesses. Biotypes AB and C are rarely isolated in liver abcesses (Berg, et al., (1982) Studies of Fusobacterium necrophorum from bovine hepatic abscesses: Biotypes, quantitation, virulence, and antibiotic susceptibility. *Am. J. Vet. Res.* 43:1580-1586), and biotype A has pathogenicity intermediate that of biotypes A and B while biotype C is non-pathogenic. (Shinjo, et al., (1990)Recognition of biovar C of Fusobacterium necrophorum (flugge) Moore and Holdeman as Fusobacterium pseudonecrophorum sp. nov., nom. rev. (ex prevot 1940) *Int. J. Sys. Bacteriol.* 41:395-397) Biotypes A and B, the most frequent types encountered in liver abscesses, have been assigned subspecies status: subsp. *necrophorum* and subsp. *funduliforme*, respectively (Shinjo et al., 1990). The subsp *necrophorum* is more virulent, produces more leukotoxin and hemagglutinin, and is more frequently isolated from cattle liver abscesses than the subsp. *funduliforme*. Virulence factors and pathogenic mechanisms contributing to the formation of liver abscesses by *F. necrophorum* are poorly understood (Tan et al., 1996). However, several studies implicate leukotoxin to be a major virulence factor (Emery, et al., (1986) Generation of immunity against Fusobacterium necrophorum in mice inoculated with extracts containing leukotoxin. *Vet. Microbiol.* 12:255-268; Tan et al., 1996). The importance of leukotoxin is evidenced by correlation between toxin production and ability to induce abscesses in laboratory animals (Coyle-Dennis, et al., (1979) Correlation between leukocidin production and virulence of two isolates of Fusobacterium necrophorum. *Am. J. Vet. Res.* 40:274-276; Emery and Vaughn, 1986), inability of nonleukotoxin-producing strains to induce foot abscesses in cattle following intradermal inoculation (Emery, et al., (1985) Culture characteristics and virulence of strains of Fusobacterium necrophorum isolated from feet of cattle and sheep. *Australian Vet. J.* 62:43-46) and relationship between antileukotoxin antibody titers and protection in experimental challenge studies (Saginala, et al., (1996a) The serum neutralizing antibody response in cattle to Fusobacterium necrophorum leukotoxoid and

possible protection against experimentally induced hepatic abscesses. *Vet. Res. Comm.*, 20:493-504; Saginala, et al., (1996b) The serum neutralizing antibody response and protection against experimentally induced liver abscesses in steers vaccinated with Fusobacterium necrophorum. *Am. J. Vet Res.*, 57:483-488; and Shinjo, et al., (1991) Proposal of two subspecies of Fusobacterium necrophorum (Flugge) Moore and Holdeman: Fusobacterium necrophorum subsp. necrophorum subsp. nov., nom. rev. (ex Flugge 1886), and Fusobacterium necrophorum subsp. funduliforme subsp. nov., nom. rev. (ex Hall 1898). *Int. J. Sys. Bacteriol.* 41:395-397).

Several investigators have attempted to induce protective immunity against *F. necrophorum* by using a variety of antigenic components. The results of such attempts have varied from ineffectual to significant protection. Clark et al. reported that cattle injected with *F. necrophorum* culture supernatant containing leukotoxin had a low incidence of foot rot caused by *F. necrophorum*. (Clark, et al. (1986), Studies into immunization of cattle against interdigital necrobacillosis. *Aust. Vet. J.* 63:107-110) Cell-free culture supernatant of a high leukotoxin producing strain of *F. necrophorum* (Tan et al., (1992) Factors affecting leukotoxin activity of *F. necrophorum. Vet. Microbiol.* 33: 15-28), mixed with an adjuvant, was shown to elicit a high antileukotoxin antibody titer when injected in steers and provided significant protection to experimentally induced liver abscesses (Saginala et al., 1996a, b; 1997). *F.necrophorum* bacterin was used as an agent for immunizing cattle and sheep against liver necrosis as shown in EPO Application No. 460480 of December 11, 1991 (the teachings of which are incorporated herein by reference). Specifically, virulent *F.-necrophorum* isolates are inactivated using ß-propiolactone, followed by addition of adjuvants. In addition, Abe et al., *Infection and Immunity*, **13**:1473-1478, 1976 grew *F.-necrophorum* for 48 hours. Cells were obtained by centrifuging, washing three times with saline, and were inactivated with formalin (0.4% in saline). The inactivated cells were then injected into mice to induce immunity. Two weeks after the last booster injection, each mouse was challenged with viable cells of *F.necrophorum.* The mice immunized with killed cells and challenged with live cells had no detectable bacteria in the liver, lung or spleen for up to 28 days. It was concluded that immunization of mice with formalin-killed *F.-necrophorum* conferred protection against infection. Garcia et al.*(Canadian J. Comp. Med,* **38**:222-226, 1974), conducted field trials to evaluate the efficacy of alum-precipitated

toxoids of *F.necrophorum*. The vaccine preparation consisted of washed cells (unlikely to contain leukotoxin) that were ruptured by sonication. The most promising result was achieved with the injection of 15.5 mg protein of cytoplasmic toxoid. In this group, the incidents of liver abscesses was reduced to 10% from an average 35% in the control group. Emery et al., *Vet. Microbiol.*, **12**:255-268, 1986, prepared material by gel filtration of 18-hour culture supernate of *F.necrophorum*. This elicited significant immunity against challenge by with viable *F.necrophorum*. The injected preparation contained endotoxin and the majority of the leukotoxic activity. U.S. Patent No. 5,455,034 (the teachings of which are incorporated herein by reference) by Nagaraja et al. disclosed that prevention of leukotoxin production (or inhibition of its activity) in immunized animals prevents the establishment of *F.necrophorum* infection. Thus, immunization of the animals against*F.-necrophorum* leukotoxin, so that the animals' white blood cells or tissue macrophages may phagocytize the bacteria, presented a way to prevent diseases associated with *F. necrophorum* infection, e.g., liver abscesses in cattle and sheep, and foot rot in cattle. In order to produce such a leukotoxoid vaccine, the *F.necrophorum* bacteria was cultured in a way to enhance the elaboration of leukotoxin in the supernate. Thereupon, bacterial growth and leukotoxin elaboration was terminated, and a vaccine prepared by inactivating at least the leukotoxin-containing supernate. In more detail, the leukotoxin elaboration method of the '034 patent involved first forming a culture of *F.necrophorum* bacteria in growth media, and thereafter causing the bacteria to grow in the culture and to simultaneously elaborate leukotoxin in the supernate. At the end of the culturing step, i.e., at the end of the selected culture time within the range of from about 4-10 hours, the bacterial growth and leukotoxin elaboration were terminated, and the leukotoxoid vaccine was prepared. This involved first separating the leukotoxin-containing supernate from the bacteria, followed by inactivation through use of formalin, ß-propiolactone, heat, radiation or any other known method of inactivation. Alternately, the entire culture could be inactivated to form the vaccine.

Presently, the control of liver abscesses is with the use of antimicrobial feed additives. Antimicrobial compounds reduce the incidence of liver abscesses but do not eliminate the problem (Nagaraja et al., 1998). Therefore, an effective vaccine would be highly desirable to the feedlot industry. The vaccine approach also would alleviate public

health concerns associated with the use of subtherapeutic levels of antibiotics in the feed. Because studies have indicated that antileukotoxin immunity reduces the incidence of hepatic abscesses and interdigital necrobacillosis (Garcia et al., 1974; Clark et al., 1986; Saginala et al., 1996a, b; 1997), the development of a recombinant leukotoxin vaccine will be of great value in the control of hepatic and interdigital necrobacillosis in cattle.

SUMMARY OF THE INVENTION

In order to better define the molecular nature of the *F. necrophorum* leukotoxin, and as a first step toward determining its specific role in the virulence of this bacterium, the leukotoxin gene was isolated, its nucleotide sequence determined, and the recombinant leukotoxin was expressed in *E. coli*.

The leukotoxin open reading frame (*lkt*A) is part of a multi-gene operon containing 9,726 bp, and encoding a protein containing 3,241 amino acids with an overall molecular weight of 335,956 daltons. *F. necrophorum* leukotoxin is highly unstable as evidenced by western blot analysis of native leukotoxin (culture supernatant, sephadex gel or affinity purified) (Figure 1). In this Figure, lane 1 contains whole cell lysate of *E. coli* cells expressing full-length recombinant leukotoxin, lane 2 contains Immuno-affinity purified native leukotoxin, lane 3 contains Sephadex gel purified leukotoxin, and lane 4 contains culture supernatant from *F. necrophorum* concentrated 60 times. The blots were probed with polyclonal antiserum raised in rabbits against affinity purified native leukotoxin. Because of the apparent instability of the full-length recombinant leukotoxin protein, the protein encoded by the gene was truncated into five recombinant polypeptides (or protein fragments, BSBSE, SX, GAS, SH and FINAL) having overlapping regions by truncating the full length gene into five different sections and amplifying, expressing in *E. coli*, and recovering the protein or polypeptide encoded by each of these sections. These polypeptides along with the full length protein are then tested to determine their immunogenicity and protective immunity in comparison to the efficacy of immunization conferred by inactivated native leukotoxin in *F. necrophorum* culture supernatant.

Specifically, the chromosomal DNA was extracted from *F. necrophorum* and partially digested by restriction endonucleases prior to being size-fractionated by sucrose gradient centrifugation. The 10-12 kb fragments were then ligated into a*Bam*HI digested,

dephosphorylated λZAP expression vector. Recombinant phages were infected into *Escherichia coli* and plated onto agar plates. Plaque lifts were performed (with polyclonal antiserum raised in rabbits against affinity purified leukotoxin) using an immunoscreening kit. Six immunoreactive recombinant phages were identified and denominated as clones 816, 611, 513, 911, 101, and 103. These clones were plaque-purified three times to ensure purity, phagemids rescued, and anti-leukotoxin immunoreactivity of the encoded proteins was confirmed. This immunoreactivity verified that the clones represented native leukotoxin *F. necrophorum.*

Expression of a polypeptide encoded by the 3.5 kb from the 5' end of the *lkt*A caused immediate cessation of the growth and lysis of *E. coli* host cells suggesting that regions of leukotoxin could be toxic to *E. coli.* Of course, the objective was to create overlapping gene truncations extending over the entire *lkt*A ORF so that the resulting polypeptide products are small and relatively stable on expression, but are large enough to be immunogenic. Also, the effectiveness of various recombinant truncated leukotoxin polypeptides alone or in combinations as immunogens and evaluated protective immunity against challenge with *F. necrophorum* in mice was investigated. The use of mice as an experimental model for *F. necrophorum* infection in cattle is well established (Abe et al., 1976; Conion et al., 1977; Smith et al., 1989; Garcia and McKay, 1978; Emery and Vaughan, 1986). Extension of the patterns of immunity and infection to cattle has shown that mice can be a valuable model to evaluate the immunogenicity and protection provided by various *F. necrophorum* fractions (Garcia et al., 1975; Garcia and McKay, 1978). Studies have also indicated that strains of *F. necrophorum* that are pathogenic in domestic animals, frequently are pathogenic in mice suggesting necrobacillosis as a disease is similar among these species of animals (Smith and Thornton, 1993).

The nucleotide sequence of the full length version of the gene is designated as SEQ ID No. 8 and the nucleotide sequences of the five truncations of the full length gene are designated as BSBSE (SEQ ID No. 9), SX (SEQ ID No. 10), GAS (SEQ ID No. 11), SH (SEQ ID No. 12), and FINAL (SEQ ID No. 13). Additionally, the nucleotide sequence of the upstream region of the full length gene is designated UPS (SEQ ID No. 14). The amino acid sequence of the full length protein encoded by the *F. necrophorum* gene is designated as SEQ ID No. 1 and the amino acid sequences of the truncated protein fragments

respectively encoded by BSBSE, SX, GAS, SH and FINAL are designated as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, and SEQ ID No. 6. In the case of UPS, the polypeptide or truncated protein fragment encoded for by UPS is designated as SEQ ID No. 7. Finally, SEQ ID No. 15 is the full length gene sequence along with contiguous sequences.

Truncated recombinant polypeptides were purified by nickel affinity chromatography, and injected into rabbits to raise polyclonal antisera. Antibodies raised against two of the five polypeptides (BSBSE and GAS) neutralized the toxicity of *F. necrophorum* leukotoxin against bovine neutrophils. The effectiveness of the purified truncated polypeptides to induce a protective immunity was determined by injecting the polypeptides, individually or in mixtures, homogenized with Ribi adjuvant in mice, followed by experimental challenge with *F. necrophorum*. Two polypeptides (BSBSE and SH) induced significant protection in mice against *F. necrophorum* infection and the extent of protection was greater than the full-length native leukotoxin or inactivated culture supernatant. The study provided further credence to the importance of leukotoxin as the major virulence factor of *F. necrophorum* and the protein carries a domain (s) or epitope (s) that induces protective immunity against experimental infection.

The DNA and deduced amino acid sequences were compared with sequences in Genbank but no significant similarities (no sequences having greater than 22% sequence identity) were found. Thus, the *F. necrophorum* leukotoxin appears to be distinct from all known leukotoxins and RTX-type toxins. When the deduced amino acid sequence of the *lktA* region was subjected to the Kyte-Doolittle hydropathy analysis (Fig. 3), 14 sites of sufficient length and hydrophobic character to be potential membrane spanning regions, were found. Upstream to the leukotoxin ORF is an open reading frame of at least 1.4 kb in length, which is in the same orientation. It encodes a protein that has significant sequence similarity (21% or 62 out of 283 residues) to the heme-hemopexin utilization protein (UxuB) of *Haemophilus infuenzae*.

Bacterial leukotoxins and cytotoxins generally have molecular masses of less than 200 kDa. This includes characterized leukotoxins of *Pasteurella hemolytica* (104,000 kDa; 10), *Staphylococcus aureus* (38,000 + 32,000 kDa; 20), or *Actinomyces actinomycetecomitans* (114,000 kDa; 15) or other pore-forming toxins of gram-negative bacteria (103, 000 to 198,000 kDa; 30). However, leukotoxin secreted by *F. necrophorum*

was shown to be approximately 300 kDa in size based on sephadex column purification and SDS-PAGE analyses.

As used herein, the following definitions will apply: "Sequence Identity" as it is known in the art refers to a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, namely a reference sequence and a given sequence to be compared with the reference sequence. Sequence identity is determined by comparing the given sequence to the reference sequence after the sequences have been optimally aligned to produce the highest degree of sequence similarity, as determined by the match between strings of such sequences. Upon such alignment, sequence identity is ascertained on a position-by-position basis, e.g., the sequences are "identical" at a particular position if at that position, the nucleotides or amino acid residues are identical. The total number of such position identities is then divided by the total number of nucleotides or residues in the reference sequence to give % sequence identity. Sequence identity can be readily calculated by known methods, including but not limited to, those described in Computational Molecular Biology, Lesk, A. N., ed., Oxford University Press, New York (1988), Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York (1993); Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H. G., eds., Humana Press, New Jersey (1994); Sequence Analysis in Molecular Biology, von Heinge, G., Academic Press (1987); Sequence Analysis Primer, Gribskov, M. et al., eds., M. Stockton Press, New York (1991); and Carillo, H., et al. Applied Math., 48:1073 (1988), the teachings of which are incorporated herein by reference. Preferred methods to determine the sequence identity are designed to give the largest match between the sequences tested. Methods to determine sequence identity are codified in publicly available computer programs which determine sequence identity between given sequences. Examples of such programs include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research, 12(1):387 (1984)), BLASTP, BLASTN and FASTA (Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990). The BLASTX program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S. et al., NCVI NLM NIH Bethesda, MD 20894, Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990), the teachings of which are incorporated herein by reference). These programs optimally align sequences using default gap weights in order to produce the highest level of sequence

identity between the given and reference sequences. As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 95% "sequence identity" to a reference nucleotide sequence, it is intended that the nucleotide sequence of the given polynucleotide is identical to the reference sequence except that the given polynucleotide sequence may include up to 5 point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, in a polynucleotide having a nucleotide sequence having at least 95% identity relative to the reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. Analogously, by a polypeptide having a given amino acid sequence having at least, for example, 95% sequence identity to a reference amino acid sequence, it is intended that the given amino acid sequence of the polypeptide is identical to the reference sequence except that the given polypeptide sequence may include up to 5 amino acid alterations per each 100 amino acids of the reference amino acid sequence. In other words, to obtain a given polypeptide sequence having at least 95% sequence identity with a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total number of amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or the carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in the one or more contiguous groups within the reference sequence. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. However, conservative substitutions are not included as a match when determining sequence identity.

Similarly, "sequence homology", as used herein, also refers to a method of determining the relatedness of two sequences. To determine sequence homology, two or more sequences are optimally aligned as described above, and gaps are introduced if

necessary. However, in contrast to "sequence identity", conservative amino acid substitutions are counted as a match when determining sequence homology. In other words, to obtain a polypeptide or polynucleotide having 95% sequence homology with a reference sequence, 95% of the amino acid residues or nucleotides in the reference sequence must match or comprise a conservative substitution with another amino acid or nucleotide, or a number of amino acids or nucleotides up to 5% of the total amino acid residues or nucleotides, not including conservative substitutions, in the reference sequence may be inserted into the reference sequence.

A "conservative substitution" refers to the substitution of an amino acid residue or nucleotide with another amino acid residue or nucleotide having similar characteristics or properties including size, charge, hydrophobicity, etc., such that the overall functionality does not change significantly.

Isolated" means altered "by the hand of man" from its natural state., i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Finally, all references and teachings cited herein which have not been expressly incorporated by reference are hereby incorporated by reference.

Preferably, sequences having at least about 50% sequence homology or at least about 60% sequence identity with any of SEQ ID Nos. 1-15 are used for purposes of the present invention. More preferably, sequences having at least about 60% sequence homology or at least about 70% sequence identity are used for purposes of the present invention. Still more preferably, sequences having at least about 75% sequence homology or at least about 85% sequence identity are used for purposes of the present invention. Even more preferably, sequences having at least about 87% sequence homology or at least about 92% sequence identity are used for purposes of the present invention. Most preferably, sequences having at least about 95% sequence homology or at least about 98% sequence identity are used for purposes of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a Western blot assay of native and recombinant leukotoxins.

Fig. 2 is an illustration of the full length *F. necrophorum* gene and a map of the truncated regions of the genes and the expression clones encoded by the truncated regions;

Fig. 3 is a Kyte-Doolittle hydropathy plot of the leukotoxin from *F. necrophorum*;

Fig. 4 is an illustration of the Southern Hybridization pattern of the chromosomal DNA of *F. necrophorum* with inserts from clones 513, 611, 816, 911, and 101;

Fig. 5 is a Kyte-Doolittle hydropathy plots of deduced amino acid sequences from the *F. necrophorum* leukotoxin gene wherein the lines above the plot correspond to the regions of the five truncated LktA polypeptides (BSBSE, SX, GAS, SH, and FINAL);

Fig. 6 is an illustration of the leukotoxin locus of *F. necrophorum*;

Fig. 7a is a Western blot analysis of truncated forms of purified recombinant leukotoxin protein probed with polyclonal antileukotoxin antiserum;

Fig. 7b is a Western blot analysis of truncated forms of purified recombinant leukotoxin protein probed with monoclonal antibody F7B10;

Fig. 7c is a Western blot of whole-cell lysates from *E. coli* clones expressing full-length recombinant leukotoxin probed with the monoclonal anti-leukotoxin antibody;

Fig. 8 is a graph illustrating the evaluation of leukotoxic activity by flow cytometry;

Fig. 9 is graph illustrating the toxicity of the recombinant leukotoxin and the truncated polypeptides by flow cytometry;

Fig. 10 is an illustration of the hybridization patterns of radiolabeled *lktA* with Southern blotted HaeIII digested restriction fragments of genomic DNAs from *F. necrophorum* subsp. *necrophorum* isolates from liver abscesses;

Fig. 11 is an illustration of the expression clones for the truncated proteins designated UPS, BSBSE, SX, GAS, SH, and FINAL;

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The following examples set forth preferred embodiments of the present invention. It is to be understood, however, that these examples are provided by way of illustration and nothing therein should be taken as a limitation upon the overall scope of the invention.

EXAMPLE 1

Cloning of the Leukotoxin Encoding *F. necrophorum* Gene

Chromosomal DNA, extracted from *Fusobacterium necrophorum subsp. necrophorum,* strain A25 (Hull et al., 1981, Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. *Infect. Immun.* 33:933-938.), was partially digested with the restriction endonuclease *Sau*3AI, and size-fractionated by sucrose gradient centrifugation (Baxter-Gabbard, 1972, A simple method for the large scale preparation of sucrose gradients. *FEBS. Lett.* 20117-119). The 10-12 kb DNA fragments were ligated in *Bam*HI-digested, dephosphorylated λZAP Express vector, packaged into lambda phage head and tail protein components (Stratagene, La Jolla, CA), and recombinant phages were infected into *Escherichia coli* XL1-Blue MRF' and plated onto agar plates. Plaque lifts were performed (with polyclonal antiserum raised in rabbits against affinity purified leukotoxin) using the Pico-blue immunoscreening kit (Stratagene, La Jolla, CA). Six immunoreactive recombinant phages were identified (816, 611, 513, 911, 101, and 103; Fig. 2). These clones were plaque-purified three times to ensure purity, and anti-leukotoxin immunoreactivity of the proteins was confirmed.

Characterization of the leukotoxin gene

Excision of the cloned DNA insert into a phagemid vector

The λZAP Express vector is composed of a plasmid, designated pBK-CMV, which flanks the cloned insert DNA and which can be readily excised in order to obtain a phagemid that contains the cloned insert DNA. Therefore, a recombinant phagemid containing cloned *F. necrophorum* DNA insert was obtained by simultaneously infecting *E. coli* XLOLR with ExAssist helper phage and the recombinant phage (containing the cloned *F. necrophorum* DNA) according to the manufacturers instructions (Stratagene, La Jolla, CA). Once the

recombinant plasmid was recovered, the presence of the DNA insert was confirmed by restriction endonuclease digestion and agarose gel electrophoresis.

Physical mapping of the F. necrophorum DNA inserts

Restriction enzyme digestion and mapping of the recombinant phagemid was performed (Sambrook et al., 1989, Molecular cloning: a laboratory manual. Cold spring harbor laboratory, Cold Spring Harbor, NY). Combinations of the restriction enzymes *Sac*I, *Sal*I, *Spe*I, *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Dra*I, *Xba*I, *Hae*III, *Bgl*II, *Sma*I, and *Kpn*I were used for restriction enzyme mapping since single sites for these enzymes exist in the multiple cloning site of pBK-CMV. Insert DNA from all the six immunoreactive clones contained *Eco*RI, *Pst*I, *Hin*dIII, *Dra*I, *Hae*III and *Bgl*II sites but not sites for *Sac* I, *Sma*I, *Sal*I, *Xba*I, *Kpn*I or *Bam*HI.

Hybridization of the Cloned DNA fragments with F. necrophorum chromosomal DNA

Southern hybridization (Southern, 1975, Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503) experiments were performed to confirm that the cloned DNA encoding the putative leukotoxin gene originated from *F. necrophorum* strain A25. Inserts from clones 513, 611, 816 and 911 were separated from the vector sequence by agarose gel electrophoresis of DNA digested with restriction enzymes *Sal*I and *Xba*I. The insert DNA was used as a probe to hybridize to chromosomal DNA of *F. necrophorum* digested with *Eco*RI, *Eco*RV, *Hae*III, and *Hin*dIII. A negative control, *E. coli* DH5α DNA, was digested with *Eco*RV. The Southern hybridization patterns included common DNA fragments indicating that the six clones carried overlapping inserts (Figure 4). Figure 2 illustrates the overlapping of each of the six immunoreactive clones designated 816, 611, 513, 911, 101, and 103. The expression clones for truncated peptides are designated UPS, BSBSE, SX, GAS, SH, and FINAL while the numbers in parentheses indicate the size in kilo-bases of each insert. The overlaps illustrated in Fig. 2 were further confirmed by sequence analysis.

DNA Sequence Analysis of the F. necrophorum DNA Inserts

Subclones of the cloned insert DNAs were constructed based on the restriction enzyme map of the cloned insert. Plasmid DNA was isolated from the resulting subclones (Birnboim and Doly, 1979, A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic acids Res.* 7:1513) and subjected to DNA sequence analysis using the Sanger dideoxy chain termination method (Sanger et al., 1977, DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci.* 74:5463-5467) using vector based primers. Additional sequence data were obtained by creating deletion clones utilizing restriction endonuclease sites discovered in the preliminary sequencing or by sequencing using primers derived from the sequenced DNA.

A total of 9.3 kb of the leukotoxin chromosomal region was cloned and sequenced. A single large open reading frame (designated *lkt*A) is common to each of the immunoreactive clones. The ORF is preceded by a ribosome binding site (RBS) sequence (AAGGGGGT). Eight base pairs following the RBS sequence is a start codon (the ninth base pair) for the open-reading frame, which is approximately 8 kb in length. The stop codon of *lkt*A was not found in this region. Therefore, the downstream sequences were extended by inverse PCR amplification, followed by cloning and sequencing of the amplified region.

Extension of the lktA Open Reading Frame Using Inverse PCR

Chromosomal DNA from *F. necrophorum* strain A25 was digested with restriction endonucleases *Taq*I, *Eco*RI, *Dde*I, or *Sau*3AI individually. After complete digestion of the chromosomal DNA with any one of these enzymes, the products were extracted with phenol and chloroform, and ethanol precipitated. Under dilute conditions (100µl final volume) 200 ng of digested DNA was self-ligated using T4 DNA ligase at 16°C overnight (Ochman et al., 1990, Amplification of flanking sequences by inverse PCR. In: M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds); PCR protocols; A guide to methods and applications. Acad. Press, Inc. Harcourt Brace Jovanovich, publishers, Sandiego, 219-227). Ligated DNA was phenol and chloroform extracted, ethanol precipitated and reconstituted in 10µl of nuclease free water. Two microliters of the ligated DNA were used as template for PCR reaction with forward and reverse primers designed based on the sequence already known to us from previous sequencing reactions. Amplified products were cloned in the

pCR 2.1 plasmid vector (Invitrogen) and sequenced using vector specific sequences. Sequencing six consecutive inverse PCR products enabled us to identify the stop codon for leukotoxin gene and the presence of another ORF downstream of *lkt*A.

The entire leukotoxin gene was amplified using heat-stable DNA polymerase (ExTaq) as two fragments using *F. necrophorum* strain A25 chromosomal DNA as the template. The 5'4.3 kb of the*lkt*A open-reading frame encoding the N-terminal half of the leukotoxin, and the 3'5.4 kb representing the C-terminal half of the leukotoxin protein. Making use of the unique Nhe I site present at this location (4.3 kb from the start codon), the leukotoxin gene was joined together to give the giant 9.726 kb ORF. The entire leukotoxin gene was cloned into the modified variant (with coding sequence for six histidine residues in the N-terminus of the expressed protein) of the expression vector pET 14b (Novagen Corp. Madison, WI). This T7 polymerase based system should enhance expression of toxic proteins, without damage to the host cell *E. coli.*

EXAMPLE 2

Preparation of Polyclonal Antileukotoxin Antiserum

Leukotoxin from *F. necrophorum* subsp.*necrophorum* strain A25 was purified using an immunoaffinity column containing antileukotoxin monoclonal antibody, F7B10 (Tan, Z. L., T. G. Nagaraja, M. M. Chengappa, J. J. Staats. 1994. Purification and quantification of *Fusobacterium necrophorum* leukotoxin using monoclonal antibodies. Vet. Microbiol. **42:**121-133.). Affinity-purified native leukotoxin (0.5 mg) in 100 µl of PBS was homogenized with an equal volume of Freund's complete adjuvant and injected intramuscularly in rabbits. A booster dose was given on day 21 with 0.5 mg of native toxin in 100 µl of PBS homogenized with an equal volume of Freund's incomplete adjuvant. Serum samples were collected on day 42. Naturally occurring rabbit antibodies that react to *E. coli* proteins were removed from the antisera as follows. Cell pellets of *E. coli* XL1-Blue MRF' host cells grown overnight in Luria broth were sonicated in PBS and centrifuged to remove cellular debris, and the supernatant was incubated with 100 mm diameter nitrocellulose membranes at 37°C for 3 hours. The nitrocellulose membranes were then washed twice in PBS-T (0.05% Tween 20 in PBS [pH 7.2]), blocked in 2% BSA, and washed three times again in PBS-T. Two ml of rabbit antileukotoxin polyclonal antiserum

were diluted 10-fold in PBS-T containing 0.2% BSA and exposed to 10 changes of *E. coli* lysate-treated nitrocellulose membranes for 30 minutes duration each at 37°C. The resultant polyclonal antisera had minimal reactivity against *E. coli* proteins. Neutralizing activity of the serum, as determined by the MTT dye neutralization test and the indirect ELISA titer, were measured as described previously (Tan, Z. L., T. G. Nagaraja, M. M. Chengappa. 1992. Factors affecting leukotoxin activity of*Fusobacterium necrophorum.* Vet. Microbiol.**33:**15-28; Tan, Z. L., T. G. Nagaraja, M. M. Chengappa, and J. S. Smith. 1994. Biological and biochemical characterization of *Fusobacterium necrophorum* leukotoxin. Am. J. Vet. Res. **55:**515-519; Tan, Z. L., T. G. Nagaraja, M. M. Chengappa, J. J. Staats. 1994. Purification and quantification of*Fusobacterium necrophorum* leukotoxin using monoclonal antibodies. Vet. Microbiol. **42:**121-133).

Extraction of Genomic Dna from *F. Necrophorum* and *E. Coli*

Chromosomal DNA was extracted from highly virulent *F. necrophorum* subsp. *necrophorum,* strain A25 (18) and *E. coli* DH5α ($F^-\lambda^-$ ϕ80 Δ [*lacZYA-argF] endA1 recA1 hsdR17deoR thi-1 supE44 gyrA96 relA1*), using a modification of the method described by Hull and coworkers (Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow. 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33:**933-938). *E. coli* was cultured in Luria broth with shaking under aerobic conditions at 37°C and *F. necrophorum* was grown overnight in a prereduced anaerobically sterilized brain heart infusion broth in serum bottles under anaerobic conditions at 39 °C. Cell pellets were resuspended in TES buffer (25% sucrose, 50 mM Tris-HCl [pH 7.5] and 1 mM EDTA); spheroplasted with lysozyme at room temperature for 30 min; and lysed using sarkosyl in the presence of proteinase K at 60°C for 1 hour. The product was extracted with buffer-saturated phenol and chloroform, and the DNA was precipitated in 2.5 volumes of ice-cold ethanol. The DNA pellet was resuspended in TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA) and subjected to ultracentrifugation in a cesium-chloride step-gradient (43.5% to 60%) containing ethidium bromide (0.4 mg/ml final volume). The chromosomal DNA band was extracted with TE buffer and CsCl saturated isopropanol to remove ethidium bromide and dialyzed against double-distilled water. The DNA concentration and purity were checked spectrophotometrically.

Genomic Library and Screening

Genomic DNA of *F. necrophorum* A25 was digested partially with restriction endonuclease *Sau*3AI, and the fragments were size-fractionated in a sucrose gradient. Ten to 12 kb fragments were cloned into *Bam*HI digested and alkaline phosphatase-treated Lambda zap Express vector (Stratagene Corp. La Jolla, Calif.) as per the manufacturer's instructions. Recombinant lambda DNA was packaged (Gigapack gold; Stratagene) and used to infect XL1Blue MRF' host cells (Stratagene). Plaques were lifted onto nitrocelluose membrane and screened with antileukotoxin polyclonal antiserum using a Picoblue immuno-screening kit as per the manufacturer's protocol (Stratagene). Immunoreactive clones were plaque purified three times using the polyclonal antiserum. The recombinant DNA from immunoreactive clones was rescued as phagemid (pBKCMV) clones using Exassist helper phage in *E. coli* XLOLR strain as per the manufacturer's protocol (Stratagene).

DNA Sequencing Analysis

Phagemids from immunoreactive clones, purified PCR products, and plasmid subclones were sequenced using vector-specific or internal primers with a model 373A automated DNA sequencer (Applied Biosystems, Foster City, Calif). The DNA sequences were aligned and analyzed using Sequencher (version 3.1.1, Gene Codes Corp., Ann Arbor, Mich) and DNA Strider (Version 1.2).

Inverse Pcr and Sequence Extension

Chromosomal DNA from *F. necrophorum* strain A25 was digested singly with restriction endonucleases *Taq*I, *Eco*RI, *Dde*I, or *Sau*3AI. After complete digestion of the chromosomal DNA with any one of these enzymes, the products were extracted with phenol and chloroform, and precipitated with ethanol. Under dilute conditions (200 ng of digested DNA in 100 μml total volume), DNA was self-ligated using T4 DNA ligase at 16°C overnight. Ligated DNA was extracted with phenol and chloroform, precipitated with ethanol and reconstituted in 10 ml of nuclease free water. Two microliters of the ligated DNA were used as templates for 100 ml PCR reactions with forward and reverse primers designed based on the sequence obtained from previous sequencing reactions. The products from inverse PCR were cloned in pCR TOPO cloning vectors (TA, Blunt2 or Blunt4) as per the manufacturer's instructions (Invitrogen Corp. San Diego, Calif.), and sequenced directly

or after subcloning, using vector specific primers. Six successive inverse PCRs were carried out to reach the 3' end of the leukotoxin gene.

Creation of Gene Truncations

Polymerase chain reaction using thermostable polymerase (EXTaq; Takara Corporation, Madison, Wis.) was used to amplify five overlapping regions of the leukotoxin gene ranging in size from 1.1 kb to 2.8 kb. Chromosomal DNA from*F. necrophorum* strain A25 was used as the template. The forward primers were designed to contain a *Sac*I site, and the reverse primers had an *Xma*I site, for in-frame insertion into the His-tag expression vector pQE30 (Qiagen Inc. Valencia, Calif.). Each truncated gene product overlapped with the adjacent product by at least 100 bp. One kb of DNA from the 3' end of the upstream open reading frame (*ups*) was amplified and cloned in pQE30 vector as described above. Recombinant plasmids were transformed into *E. coli* host strain M15 for inducible expression of proteins encoded by cloned genes under the control of the *lac* promoter. The five truncated leukotoxin polypeptides and the C-terminus of the upstream polypeptide were purified using nickel chelation chromatography under denaturing conditions to apparent homogeneity as indicated by silver-stained SDS-PAGE gels (data not shown).

Preparation of Polyclonal Antiserum Against the Truncated Leukotoxin Polypeptides

New-Zealand White rabbits were injected intramuscularly with the five truncated leukotoxin polypeptides or the upstream polypeptide (0.5 mg/animal) precipitated with aluminum hydroxide. A booster dose was given on day 21 (0.5 mg /animal). Serum samples were collected on days 21 and 42 and antileukotoxin titers were determined by indirect ELISA using affinity purified native leukotoxin (Tan, Z. L., T. G. Nagaraja, M. M. Chengappa, J. J. Staats. 1994. Purification and quantification of *Fusobacterium necrophorum* leukotoxin using monoclonal antibodies. Vet. Microbiol. **42:**121-133.). Leukotoxin neutralizing activities of the 42 day serum samples were determined by the MTT dye neutralization assay using 200 units of toxin (*id.*).

Immunoblot Analysis

Affinity-purified native leukotoxin, the truncated leukotoxin polypeptides and upstream polypeptide purified over nickel columns, whole cell lysates from bacterial clones carrying recombinant expression plasmids, and concentrated culture supernatants were resolved by SDS-PAGE (6 or 10% acrylamide) and electroblotted to nitrocellulose membranes (BioRad minigel II electrophoresis and transfer unit). Monoclonal antibody against native leukotoxin (F7B10) or polyclonal antisera raised against native leukotoxin, various truncated leukotoxin or upstream polypeptides were used to probe the western blotted proteins. Goat antimouse or antirabbit IgG conjugated to alkaline phosphatase (Sigma Chemical Company, St. Louis, MO) was used as the secondary antibody, and the immunoreactive proteins were detected using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as substrates.

Cloning and Expression of Full-length Leukotoxin ORF

A 4.3 kb DNA fragment containing the 5' end of the *lktA* open reading frame up to the internal NheI restriction endonuclease recognition site was amplified from A25 chromosomal DNA. This fragment was cloned into the kanamycin resistance encoding vector pCR Blut II TOPO. A 5.4 kb DNA fragment extending from the NheI site to the 3' end of the *lktA* open reading frame was PCR amplified and cloned into the low-copy, spectinomycin resistance plasmid pCL1921 (Lerner, C. G., and M. Inouye. 1990. Low copy number plasmids for regulated low level expression of cloned genes in *Escherichia coli* with blue/white insert screening capability. Nucl. Acid. Res.**18:**4631-4633.). The two resulting plasmid clones were ligated together making use of the unique *Nhe*I site present in *lkt*A ORF, and the transformants were selected on media containing spectinomycin (100 μg/ml) and kanamycin (21 μg/ml). The pCR Blunt II vector specific sequences were then removed by digesting the resultant plasmid with *Sac*I followed by ligation under dilute conditions and selection on L-agar containing 100 μg/ml spectinomycin. Thus the entire 9,726 base pairs of the leukotoxin ORF were cloned in a low-copy number plasmid pCL1921 to produce pSN1999. Making use of the unique *Xma*I site introduced into at the 3' end of the open reading frame and the *Sac*I site introduced into the 5' end of the reading frame, the entire *lkt*A coding sequence was cloned in-frame into the expression plasmid pQE30 to give pSN2000.

Flow Cytometric Analysis of Leukotoxin Biological Activity

Bovine peripheral polymorphonuclear leukocytes were isolated as described previously (Tan, Z. L., T. G. Nagaraja, M. M. Chengappa. 1992. Factors affecting leukotoxin activity of *Fusobacterium necrophorum.* Vet. Microbiol. **33:**15-28; Tan, Z. L., T. G. Nagaraja, M. M. Chengappa, and J. S. Smith. 1994. Biological and biochemical characterization of *Fusobacterium necrophorum* leukotoxin. Am. J. Vet. Res**55:**515-519). Untreated cells (negative control) or those treated with either 200 units of native leukotoxin from *F. necrophorum* (positive control) or whole-cell lysates from clones expressing full-length recombinant leukotoxin were tested for viability by flow cytometry (Facstar, Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Briefly, 1 ml of bovine peripheral PMNs ($9x10^6$ cells/ml) was incubated with various preparations of toxin for 45 min at 37°C in a chamber containing 5% CO_2. The cells were then washed twice in 2 ml of HBSS (pH 7.2) and resuspended in 300 µl of HBSS. These cells were treated for 10 min in the dark at room temperature with 10 µl of 5 mg/ml propidium iodide (PI). The red fluorescence (FL-2 [585/42]) is proportional to the number of cells which have lost membrane integrity and, therefore, do not exclude the propidium iodide. Leukocyte subpopulations were displayed in a dot plot and gated according to size based on forward scatter (FSC) and granularity or 90 degree light scatter (SSC). A region was placed around granulocytes, cells of larger size and granularity and thus excluding monocytes, and data were collected on 10,000 gated cells. The identity of the gated cells as granulocytes by was indicated by indirect immunofluorescence labelling with monoclonal antibody DH59B (VMRD Inc., Pullman, Washington) which reacts with the granulocyte-monocyte-1 receptor. Fluorescence signals displayed as a dot plot were used to determine the percent positive cells by quadrant statistics.

Southern Blot Analysis

Genomic DNA was extracted from several strains of *F. necrophorum* subsp. *necrophorum* and subsp. *funduliforme* isolated from ruminal contents or liver abscesses. Chromosomal DNA was digested to completion with *Hae*III, which cleaves the leukotoxin ORF once. The digested DNA was electrophoresed in a 1% agarose gel and Southern

blotted onto a nitrocellulose membrane. The full-length *lktA* ORF cloned in pQE30 (pSN2000) was released by digestion with *Sac*I and *Xma*I, and the insert DNA was gel purified, radiolabelled with [α-^{35}S]dATP, and hybridized.

Nucleotide Sequence Accession Number

The nucleotide sequence of *F. necrophorum* subsp. *necrophorum* strain A25 *lktA* has been assigned GenBank accession number AF312861.

Cloning and Nucleotide Sequence of the *F. Necrophorum* Leukotoxin Determinant

A Sau3A-generated genomic library of *F. necrophorum* strain A25 DNA was screened using rabbit polyclonal antisera raised against immunoaffinity-purified native leukotoxin and immunoreactive clones were identified. The clones carried inserts of approximately 4.6, 5.5, and 6.3 kb in length. The immunoreactive clones containing the leukotoxin open reading frame (designated *lktA*) are depicted in Figure 1. Inverse PCR was used to extend the cloned region to allow completion of the sequence of the *lktA* open reading frame. The 11,130 bp sequence of *F. necrophorum* DNA contained one complete and two partial ORFs. The upstream (*orfB*) partial ORF comprises the first 1,018 bp. The *lktA* ORF initiates 16 bp downstream of the *lktB* ochre codon. A putative ribosome-binding site (RBS) with the sequence AAGGGGGT precedes the *lktA* ORF. The first two bases of the RBS were the last two bases of the *lktB* stop codon. The leukotoxin determinant is 9,726 bp and encodes a protein of 3,241 amino acids with an overall molecular weight of 335,956. The deduced protein sequence is unusual in that it lacks cysteine residues. The protein has substantial hydrophobic character (Fig. 5) and possesses 14 regions with sufficient hydrophobic character and length to be membrane spanning. However, this is a secreted toxin in *F. necrophorum*. The potential transmembrane domains may provide a clue as to the mode of action of the leukotoxin on the target neutrophils.

A BLAST search of the protein database with the deduced leukotoxin did not indicate significant sequence similarity to any bacterial cytotoxins. Some sequence similarity, generally 17-20% amino acid identity over a window of 1,500 to 2,000 residues, was found to certain high molecular weight cell surface proteins. These include the SrpA serine-rich protein from *Streptococcus cristatus* (accession number U96166), the

hemagglutinin from *Streptococcus gordonii* (AB029393), a surface protein from *Xylella fastidiosa* (AE003982), the outer membrane protein A from *Rickettsia australis* (AF149108), the 190 kDa surface antigen precursor from *R. rickettsii* (A41477), and the high molecular weight antigen (HmwA) of *Haemophilus influenzae* (AF180944). Given the molecular size of the leukotoxin protein, which is larger than any known bacterial exotoxin, its lack of cysteine residues, and its lack of sequence similarity to other bacterial leukotoxins, the LktA protein from *F. necrophorum* appears to be a novel leukotoxin.

The deduced amino acid sequence of the carboxy terminus of the OrfB protein has some sequence identity to heme-hemopexin utilization protein (HxuB) of *Haemophilus influenzae* (21% amino acid identity over a 283 residue window). The putative open reading frame upstream of the leukotoxin determinant does encode a protein product. The 1 kb sequence encoding the carboxyl terminus of this ORF was cloned into pQE30, and the polypeptide was expressed with the six histidine tag at its N-terminus. The protein was purified by nickel chelation chromatography, and the antiserum was raised against this protein in rabbits. Western blot analysis revealed that this antiserum recognized a 60 kDa protein in whole-cell lysates of *F. necrophorum* (data not shown). This protein was not present in culture supernatants or in purified outer membranes *of F. necrophorum*.

Downstream of *lktA* is another apparent open reading frame, which extends to the end of the cloned sequences (375 bp). The putative ATG start codon overlaps the opal stop codon of *lktA*. The nucleotide and deduced amino acid sequences do not show significant sequence similarity to any sequences currently in GenBank.

Creation of Truncated Leukotoxin Polypeptides and Characteristics of Polyclonal Antisera Raised Against Them

A 3.5 kb sequence from the 5' end of *lkt*A gene was amplified by PCR and cloned in-frame in the expression vector pQE30. Induced expression of this truncated version of the leukotoxin protein with IPTG resulted in the immediate cessation of growth and lysis of the host *E. coli* cells. In order to obtain better expression of recombinant protein and less toxicity to *E. coli* host cells, smaller truncations of the leukotoxin gene were constructed. The truncated polypeptides were named BSBSE, SX, GAS, SH, and FINAL starting from the N-terminus and ending at the C-terminus of the leukotoxin protein (Fig. 6). In this

Figure, the boxes represent the leukotoxin open reading frame (*lktA*) and its flanking putative open reading frames. The lines above the boxes represent the phagemid clones (816, 101, and 611) obtained from the immunoreactive plaques in the cloning experiments. The region designated iPCR represents the sequence obtained from sequencing a series of inverse PCR clones. The plasmid pSN2000 contains the entire *lktA* open reading frame. Below the boxes are the clones expressing the truncated leukotoxin polypeptides. The numbers refer to the nucleotide positions of the boundaries of each truncation relative to the 11,130 bp sequence deposited in GenBank.

Each polypeptide had an overlap of at least 21 amino acids with its adjacent polypeptide. The C-terminal truncated polypeptide of the upstream protein and the polyclonal antiserum raised against it, served as a negative control in our toxicity and toxin-neutralization studies. Purified truncated leukotoxin and upstream polypeptides were then analyzed by western blots, for their reactivity against polyclonal and monoclonal antisera raised against affinity-purified native leukotoxin, using western blot analysis. Antileukotoxin polyclonal antisera reacted strongly with polypeptides BSBSE, SX, and FINAL and weakly with polypeptides GAS and SH (Fig. 7a). Monoclonal antileukotoxin antibody reacted with the N-terminal polypeptide, BSBSE, but not any other truncated leukotoxin polypeptides (Fig. 7b). As expected, the UPS polypeptide did not react with polyclonal or monoclonal antileukotoxin antibodies. Polyclonal antisera raised in rabbits against each of the truncated leukotoxin polypeptides reacted strongly with the corresponding polypeptide and also the native leukotoxin. These results are shown below in Table 1. Antibodies raised against individual truncations reacted weakly to their adjacent polypeptides because of the presence of the overlapping amino acid sequences between them (data not shown). Antiserum raised against UPS (from the upstream ORF) failed to recognize the leukotoxin.

Table 1. Neutralization of Leukotoxin from *F. Necrophorum* by Rabbit Polyclonal Antisera Raised Against the Recombinant Truncated Polypeptides.

Immunogen	ELISA Titer		Neutralization Titer
	Self polypeptide	Native Leukotoxin	
UPS	9,600±1,693	19±17	<5
BSBSE	10,420±1,142	10,680±1,653	1,460 ±71
SX	8,754±983	7,480±1,593	<5
GAS	8,748±865	8,100±1,297	1,280±89
SH	10,180±1,789	8,220±1,301	<5
FINAL	9,750±1,343	9,440±1,262	<5

ELISA titers are presented as the mean of three determinations expressed as the reciprocal of the highest dilution giving a positive reaction (± standard deviation). The neutralization titer is the reciprocal of the greatest dilution of antiserum able to neutralize the activity of 200 units of native leukotoxin in an MTT assay.

Antisera raised against the individual polypeptides were tested for neutralization activity against the native leukotoxin from *F. necrophorum*. An ELISA assay was utilized to measure the reactivity of each antiserum against the leukotoxin. An MTT dye reduction assay was then utilized to determine if the antiserum could neutralize the toxic effects of the leukotoxin against bovine peripheral leukocytes. As shown in Table 1, two of the antisera could neutralize the leukotoxin. The active antisera were raised against the N terminal polypeptide (BSBSE) and the middle polypeptide (GAS). The other three antisera did not have neutralizing activity in this assay, although the ELISA data indicated that each antiserum recognized the *F. necrophorum* leukotoxin.

Creation of Full-length Recombinant Leukotoxin and its Toxicity to Bovine Peripheral Blood Polymorphonuclear Cells

The entire leukotoxin gene (9,726bp) was cloned into the pQE30 expression vector. Unlike certain truncated versions of the leukotoxin protein, full-length recombinant

leukotoxin upon expression was not toxic to *E. coli* host cells. When whole-cell lysates from clones expressing full-length leukotoxin were subjected to western blot assays, both polyclonal (not shown) and monoclonal antileukotoxin antibodies reacted to high-molecular weight (>220 kDa) protein species (Fig. 7c). In this Figure, MW is molecular weight markers; Lkt, is affinity-purified leukotoxin from *F. necrophorum*; FL-I and FL-UI are full-length clone induced or uninduced with IPTG; Super is concentrated *F. necrophorum* A25 culture supernatant. Additionally, the arrows denote the positions of the reactive BSBSE band in Fig. 7b and the full-length leukotoxin in Fig. 7c. The amount of full-length leukotoxin in the culture supernatant in panel C was insufficient to be visualized as a distinct band in this blot. The protein was extremely unstable, as evident by the presence of numerous smaller molecular weight species, which presumably represent breakdown products. This instability was also observed with native leukotoxin that was immunoaffinity-purified from *F. necrophorum* culture supernatants. Antisera raised against all the truncated leukotoxin polypeptides, including the C-terminal FINAL polypeptide, reacted to recombinant leukotoxin suggesting that the protein may be expressed in its full-length (data not shown). As expected, antibody raised against the upstream polypeptide failed to react to the full-length recombinant leukotoxin.

Bovine peripheral polymorphonuclear leukocytes exposed to whole-cell lysates of full-length or truncated recombinant clones (12 mg/ml protein) prior to or after induction with IPTG were tested for membrane integrity using propidium iodide exclusion and flow cytometry. Control cells untreated with leukotoxin gave a baseline value of 5.4% PI-staining cells (Fig. 8). In this Figure, membrane damage was assessed by staining of the cells with propidium iodide. Shown are the values obtained after counting 10,000 PMNs (stippled bars) or the lymphocyte fraction (hatched bars). Cells were untreated (control), treated with 200 units of affinity purified leukotoxin from *F. necrophorum* (Fn leukotoxin) or lysates of *E. coli* harboring expression plasmids bearing the upstream polypeptide (pSN100) or the full-length *lktA* open reading frame (pSN2000). The "U" and "I" designations refer to lysates from uninduced cultures and cultures induced with 1 mM IPTG, respectively. Induced lysates were also tested after 1:5, 1:25, and 1:125 dilutions in PBS. The results shown are the averages of three experiments and the standard deviation is indicated.

The addition of 200 MTT units of affinity-purified native leukotoxin resulted in 75.4% of the PMNs taking up the dye. An MTT unit of the toxin is defined as the reciprocal of the dilution causing a 10% decrease in MTT-dye reduction activity. The affinity-purified leukotoxin preparation used in this study had an activity of $2x10^5$ units/ml. Lysates from the clone expressing the upstream polypeptide (SN100) did not increase the percentage of PI-staining cells, indicating that the truncated form of this protein lacked membrane-damaging activity. Whole-cell lysates from *E. coli* carrying recombinant full-length leukotoxin gene (SN2000), uninduced with IPTG, gave rise to 9.6% PI-staining bovine PMNs, whereas lysates from induced clones gave 27.3% staining PMNs. The low percentage of damaged cells from the uninduced lysate resulted from leaky expression of the toxin with this vector, consistent with the results obtained by western blot analysis (not shown). The membrane damaging activity in the induced lysate was proportionately lost when the samples were diluted in phosphate-buffered saline. The data indicate that recombinant full-length leukotoxin is toxic to bovine neutrophils.

Preparations of PMNs had residual contaminating cells of smaller size and granularity, which were found to be predominantly lymphocytes by immunophenotyping with anti-CD3 and anti-IgM specific monoclonal antibody. These cells were gated, and the effects of various leukotoxin preparations on the viability of these cells were measured as described for PMNs. Untreated control lymphocytes gave a baseline value of 13.6% staining cells, whereas inclusion of 200 units of affinity-purified native leukotoxin resulted in 31.3% of the lymphocytes taking up the PI (Fig. 8). The apparently lower sensitivity of lymphocytes compared to PMNs is characteristic of *F. necrophorum* leukotoxin. Furthermore, the recombinant toxin displayed the same degree of activity against lymphocytes as did the native leukotoxin. Among lymphocytes treated with lysates from *E. coli* carrying uninduced recombinant full-length *lkt*A, 12.8% were PI-positive compared to 19.2% obtained with lysates from induced clones. Thus the expressed recombinant leukotoxin had toxicological properties similar to those of the native leukotoxin purified from *F. necrophorum* culture supernatant. Lysates from *E. coli* with IPTG-induced expression of the leukotoxin truncated polypeptides or the upstream polypeptide did not display membrane-damaging activity against either bovine PMNs or the lymphocyte-containing population (Fig. 9). In this Figure, membrane damage was assessed by staining

of the cells with propidium iodide. Shown are the values obtained after counting 10,000 PMNs (stippled bars) or the lymphocyte fraction (hatched bars). Cells were untreated (control), treated with 200 units of affinity purified leukotoxin from *F. necrophorum* (native toxin), lysates from IPTG-induced cultures of clones expressing the truncated polypeptides (ups, BSBSE, SX, GAS, SH, and Final) or the whole recombinant leukotoxin (whole toxin). The results shown are the averages of three experiments and the standard deviation is indicated.

Presence of the Leukotoxin Determinant in *F. Necrophorum* Isolates

The leukotoxin gene was cloned and sequenced from *F. necrophorum* subsp. *necrophorum* A25, a strain originally isolated from a bovine liver abscess. Southern blot hybridization of the chromosomal DNA extracted from various *F. necrophorum* strains of both subspecies isolated from ruminal contents or liver abscesses was carried out using the leukotoxin ORF as a probe (Fig. 10). In this Figure, *F. necrophorum* subsp. *necrophorum* from liver abscesses are in lane 1 which is strain A21; lane 2 which is A25; and lane 3 which is A39. *F. necrophorum* subsp. *necrophorum* from ruminal contents are in lane 7 which is RA13; lane 8 which is RA15; lane 9 which is RA16; lane 10 which is RA18; lane 11 which is RA26; lane 12 which is RA28; and lane 13 which is RA29. The *F. necrophorum* subsp. *funduliforme* isolates from liver abscesses are in lane 4 which is B17; lane 5 which is B29; lane 6 which is B35 or ruminal contents which are in lane 14 which is RB33; and lane 15 which is RB37. Strains are described in reference 24. M, DNA molecular weight markers. The restriction endonuclease *Hae*III was used to digest the chromosomal DNA from *F. necrophorum* isolates. A single recognition site for this enzyme occurs 5,933 bp from the start codon in the *lkt*A ORF. Thus, two hybridizing fragments should be present in strains carrying this gene. All strains of *F. necrophorum* subsp. *funduliforme* isolated from liver abscesses (B17, B29, and B35) or ruminal contents (RB33 and RB37) were identical in their hybridization patterns showing two bands at approximately 7 and 8 kb each. Also, all isolates of*F. necrophorum* subsp. *necrophorum*, except A39, isolated from liver abscesses (A21 and A25) and those isolated from ruminal contents (RA13, RA15, RA16, RA18, RA26, RA28, and RA29) had identical hybridization patterns showing two bands of approximately 10 and 11kb each. A single band of approximately 10.5 kb,

presumably a doublet, hybridized to the leukotoxin gene in chromosomal DNA of strain A39 (Fig. 10, lane 4). This suggests that some heterogeneity may be present in the leukotoxin locus sequences among strains of *F. necrophorum* subsp. *necrophorum*. However, the hybridization pattern does appear to be a good indicator for subspecies determination.

5

EXAMPLE 3

Construction of Truncated Forms of the Leukotoxin

A 3.5 kb sequence from the 5' end of lktA gene was amplified by PCR and cloned in-frame in the expression vector pQE 30 (Qiagen Corporation). Induced expression of this
10 truncated version of the leukotoxin protein with IPTG resulted in the immediate cessation of growth and caused lysis of the host *E. coli* cells. In order to obtain better expression of recombinant protein, smaller truncations of the leukotoxin gene were constructed. Polymerase chain reaction using thermostable polymerase with proof reading ability (EXTaq; Takara Corp.) was used to amplify five overlapping regions of the leukotoxin gene.
15 The forward primers were designed to contain a *Sac*I site, and the reverse primers had a *Xma*I site. *F. necrophorum* A25 chromosomal DNA was used as the template, and the amplified products were digested with restriction enzymes *Sac*I and *Xma*I, and cloned in-frame in the His-tag expression vector pQE 30. Five truncated leukotoxin proteins and the C-terminus of the upstream protein were purified using nickel chelation chromatography
20 to apparent homogeneity as indicated by silver-stained SDS-PAGE gels. The proteins were then tested for their reactivity with polyclonal antisera raised in rabbits against affinity purified native leukotoxin using western blot analysis. Purified proteins were injected in rabbits to produce polyclonal antisera, which in turn were used to carry out western blot analysis and neutralization tests (Table 2). Antisera raised against each protein recognized
25 native leukotoxin from *F. necrophorum*. Antisera directed against the BSBSE9 and GAS polypeptides were able to neutralize the activity of native leukotoxin. Thus the cloned ORF does indeed represent the *F. necrophorum* leukotoxin.

30

Table 2. Characterization of the Truncated Upstream and Leukotoxin Proteins

Truncated Leukotoxin Proteins (N to C terminal)	Number of Amino Acids	Size (in Daltons)	Recognized by Anti-native Leukotoxin Antibodies	Antisera Raised Against Truncated Proteins Recognized Native Leukotoxin	Antisera Neutralizes Activity of Leukotoxin Against PMNs
UPS 9	339	38324	–	–	–
BSBSE 9	377	40810	+	+	+
SX7	926	97453	+	+	–
GAS 15	713	71949	–	+	+
SH 12	628	63457	–	+	–
FINAL 2	774	80590	+	+	–

Production of an Inactivated Recombinant Leukotoxin Vaccine

The immunogenicity and protective immunity of the recombinant full length and truncated leukotoxin proteins is determined in mice and compared to the efficacy of immunization with inactivated native leukotoxin in *F. necrophorum* culture supernatant. The usefulness of the mouse model in studying experimental *Fusobacterium* infections has been well documented (Abe et al., 1986, Emery and Vaughn, 1986).

Vaccine Preparations

Purified recombinant leukotoxins (described above) including the full-length protein are inactivated by the addition of formalin (final concentration 0.3%) and homogenized with Ribi or other suitable adjuvant (10% vol/vol; Ribi Immunochem, Hamilton, MT). The native leukotoxoid vaccine is prepared with culture supernatant from *F. necrophorum* subsp. *necrophorum*, strain A25 grown in PRAS-BHI broth (Saginala et al., 1997). The leukotoxic activities of the recombinant leukotoxin and culture supernatant, before and after formalin

inactivation, are then tested by MTT-dye reduction assay using bovine polymorphonuclear (PMN) leukocytes as target cells (Tan et al., 1992). The quantity of native leukotoxin is then assayed using a sandwich ELISA using purified monoclonal antibody (Tan et al., 1994b).

Immunogenicity of the Inactivated Recombinant Leukotoxin in Mice

Immunogenicity and protective effects of the inactivated recombinant full length, and truncated leukotoxins are evaluated in comparison with the native leukotoxin (culture supernatant of *F. necrophorum*, strain A25). Five overlapping truncations and the recombinant full-length leukotoxin are purified using the nickel-affinity columns. The treatment groups include control (0.2 ml PBS), native leukotoxin, recombinant full length, and truncated leukotoxins individually or in combination (all five truncations individually, and a mixture of all five truncated proteins in equimolar ratio). Additionally, a mixture of the two truncated proteins BSBSE and GAS in equimolar concentrations is tested for immunogenicity, because polyclonal antisera raised against these two proteins neutralize the activity of native leukotoxin against bovine neutrophils. Each leukotoxin preparation is tested at 10 and 50 μg doses (total protein concentration), administered subcutaneously on days 0 and 21. Six mice (7-8 wk old BALB/c) are used in each treatment group. Blood samples are collected on days 0, 14, 21, 35, and 42. Serum is stored at -70°C until assayed for antileukotoxin antibody. After the last blood sampling (on day 42), mice are challenged intraperitoneally with 0.4 ml of late-log phase *F. necrophorum* strain A25 culture (6-7 hour culture in PRAS-BHI broth with an absorbance of 0.65 at 600 nm and with a cell concentration of approximately 1 to 5 x 10^8 CFU/ml). The number of bacteria used for inoculation is enumerated by viable counts on blood agar plates in an anaerobic glove Box (Forma Scientific, Marietta, OH). Mice are observed for 4 days after challenge to record mortality and clinical signs, and those that survive the challenge are euthanized. Mice are then necropsied and examined grossly for abscesses in the liver. Additionally, other organs and liver tissue will be cultured for anaerobic bacterial isolation.

Following this study, the efficacious dose and the recombinant leukotoxin preparation is selected and one more immunization and challenge study in mice to confirm the protective effect of recombinant leukotoxin is conducted. Groups of 7-8 week old BALB/c mice (10 per group) are used and each group receives one of the following

leukotoxin preparations: most immunogenic recombinant leukotoxin protein, combination (two or more) of most immunogenic recombinant leukotoxin proteins, and native leukotoxin (*F. necrophorum* culture supernatant). The leukotoxin proteins are inactivated with 0.3% formalin, mixed with Ribi or any other suitable adjuvant and emulsified with a homogenizer and administered subcutaneously on days 0 and 21. Blood samples are collected on days 0, 14, 21, 35 and 42. Serum samples are assayed for antileukotoxin antibody. After the last blood sampling (on day 42), mice are challenged as described above. Overlapping variants of effective polypeptides (the truncated protein fragments) are identified and are constructed in order to identify the polypeptide sequences that are most effective in conferring protection.

Determination of Antileukotoxin Antibody Induced by Immunization

Mouse serum is analyzed for antileukotoxin antibody by two methods. First, serum samples are assayed for leukotoxin neutralizing antibody by testing its ability to neutralize the toxin using the MTT dye reduction assay with mouse and bovine PMNs as the target cells (Saginala, et al., 1996b; Tan et al., 1994a). Second, serum samples are tested for anti-leukotoxin IgG antibodies by enzyme linked immunosorbent assay (ELISA) using affinity-purified leukotoxin as the coating antigen. Affinity purification of the leukotoxin is carried out using monoclonal antibody MAbF7B10 (Tan et al., 1994b).

EXAMPLE 4

DNA extraction and polymerase chain reaction

Chromosomal DNA was isolated from *F. necrophorum* subspecies *necrophorum,* strain A25. Briefly, *F. necrophorum* was grown overnight in a PRAS-BHI broth in serum bottles at 39°C. Cell pellets were resuspended in TES buffer (25% sucrose, 50 mM Tris-HCl [pH 7.5] and 1 mM EDTA), spheroplasted with lysozyme at room temperature for 30 min, and lysed using sarkosyl in the presence of proteinase K at 60°C for 1 hour. The DNA was extracted with buffer-saturated phenol and chloroform and was precipitated in 2.5 volumes of ice-cold ethanol and 1/10 volume of sodium acetate (3 M, pH 5.2). The DNA pellet was resuspended in TE buffer (10 mM Tris-HCl [pH 8.0] and 1mM EDTA) and was run for 20 hours in a cesium-chloride gradient (60% to 43.5%) containing ethidium bromide (0.4

mg/ml final volume). The chromosomal DNA band was extracted with cesium-chloride saturated isopropanol to remove ethidium bromide and dialyzed against double distilled water. DNA concentration and purity were checked spectrophotometrically.

The primers were designed to amplify the leukotoxin gene as five overlapping truncations (Table 3). The sites for annealing of the primers were chosen, so that there is an overlap of approximately 100 bp with the adjacent truncated leukotoxin gene product. Each forward primer was designed to contain a *Sac*I site and reverse primers carried a *Xma*I site (Table 3). PCR amplifications were carried out under following conditions using a thermostable DNA polymerase with a proof-reading function ExTaq (Takara Corp., Madison, Wisconsin): initial denaturation 94°C for 3 min; 36 cycles of denaturation 94°C for 1 min, 59°C for 45 sec, 67°C for 30 sec, and 72°C for 1 to 3 min (at min per kb), and a final extension at 72°C for 4 min.

Table 3. PCR primers used for amplifying truncated leukotoxin gene segments.

Truncated segment	Location in lktA gene (bp)	Designation	Primer Sequence[a]
bsbse	1-22	BS-START	tccgagctcATGAGCGGCATCAAAAATAACG
	1130-1112	BS-END	tcgccccgggATAGGAGAAATAGAACCTG
sx	919-940	SX-START	tccgagctcGGGAGATTTATAAAGAAAGAAG
	3698-3679	SX-END	tcgccccgggGATCCGCCCCATGCTCCAAC
gas	3553-3572	GAS-START	tccgagctcGGAGCTTCTGGAAGTGTTTC
	5693-5674	GAS-END	tcgccccgggGTACTATTTTTTATATGTGC
sh	5623-5641	SH-START	tccgagctcGCTGCAGTAGGAGCTGGAG
	7510-7492	SH-END	tcgccccgggCTGCAGTTCCCAAACCACC
final	7405-7425	FIN-START	tccgagctcGGAATTAAAGCCATTGTGAAG
	9726-9706	FIN-END	tcgccccgggTCATTTTTTCCCTTTTTCTCC

[a]Lower case letters in primer sequences represent extra bases added to incorporate restriction sites.

Directional cloning in an expression vector

The amplified gene products which are overlapping truncations extending from 5' to 3' end of the leukotoxin gene (*lkt*A), were named BSBSE, SX, GAS, SH, and FINAL (Fig. 11). In this Figure the numbers in parentheses indicate the size in kilobases of each insert. They were extracted with phenol and chloroform and precipitated with ethanol as described above. The amplified *lkt*A gene products and expression vector pQE30 (Qiagen Corp., Valencia, California) were digested with restriction endonucleases *Sac*I and *Xma*I as per manufacturer's instructions (New England Biolabs, Beverly, Massachusetts). After digestion, the vector and insert DNA were phenol and chloroform extracted, ethanol precipitated, and ligated overnight at 16°C using T4 DNA ligase (Promega Corp., Madison, Wisconsin). Ligated DNA was digested with restriction enzyme*Kpn*I before transforming chemically competent *E. coli* M15 cells as per standard procedures. Restriction sites for *Kpn*I is absent in the entire *lkt*A gene and present in a single location between *Sac*I and *Xma*I sites in pQE 30. The expression vector pQE 30 lacks blue/white selection, thus the above manipulation helped us to enrich clones that carry truncated leukotoxin gene products. The transformants were plated on Luria-agar plates containing ampicillin (100 ug/ml) and kanamycin (20 ug/ml) to select for clones containing plasmids pQE30 and pRep4.

Expression of truncated leukotoxin polypeptides

Plasmid DNA from the transformants were purified using Wizard SV miniprep columns (Promega), and the orientation of the insert was checked by sequencing with a vector specific 5'QE primer which anneals upstream to the MCS using a Applied Biosystems 373A automated sequencer. Positive clones were induced for the expression of polypeptides with IPTG, the whole cell lysates from uninduced and induced were compared for immunoreactive polypeptides in a western-blot using polyclonal antisera raised in rabbits against affinity purified native leukotoxin (Tan et al, 1994d).

Antigen preparation

Due to the presence of its codons in the sequence upstream of the MCS in the vector pQE 30, six histidine residues are added in the N-terminus of the expressed polypeptides. The expressed polypeptides were purified using nickel-affinity columns under denaturing

conditions using guanidium hydrochloride, as per the manufacturer's instructions (Qiagen). The column purified polypeptides were dialyzed for 48 hours at 4°C against sterile phosphate buffered saline (0.1 M, pH 7.2) to remove any traces of urea, and concentrated in Ultrafree-Biomax 30 filters (Millipore Corp. Bedford, Massachusetts), which retains molecules of sizes over 30 kDa. The protein concentrations were analyzed using the BCA assay (Pierce, Rockfort, Illinois) and the purity checked with SDS-PAGE analysis followed by silver staining. Native leukotoxin from*F. necrophorum* culture supernatant was purified using immunoaffinity columns with anti-leukotoxin monoclonal antibody (F7B10) as described previously. Also, leukotoxoid vaccine (12 hours culture supernatant inactivated with 0.3% formaldehyde) was made as described previously (Saginala et al., 1997).

Preparation of polyclonal antiserum against truncated polypeptides

Five New-Zealand White rabbits were injected intramuscularly with the five truncated leukotoxin polypeptides (0.5 mg/animal) precipitated with aluminum hydroxide. A booster dose was given on day 21 (0.5 mg /animal). Serum samples were collected on days 21 and 42 and antileukotoxin titers were determined by indirect ELISA using affinity purified native leukotoxin. Leukotoxin neutralizing activities of the 42 day serum samples were determined by the MTT dye neutralization assay. A neutralization ratio, which was the dilution of the antiserum that caused neutralization divided by its ELISA titer, was calculated for each truncated polypeptide.

EXAMPLE 5

Vaccine and Immunization

One hundred (100) 8 to 10 week old mice, identified by ear-markings, were randomly divided into 10 groups of 10 mice each. The groups received five truncated leukotoxin polypeptides (BSBSE, SX, GAS, SH, and FINAL) individually, a mixture of BSBSE and GAS, a mixture of all five truncated polypeptides, affinity purified native leukotoxin, inactivated culture supernatant, or PBS emulsified with Ribi adjuvant. Each mouse was injected subcutaneously (in two locations of 100 µl each between the shoulder blades) on day 0 and day 21 with 200 µl of one of the above preparations. The total amount of antigen in each injection (except with culture supernatant or PBS) was 10 µg per animal.

Inactivated culture supernatant (12 mg/ml protein concentration) was used without dilution to reconstitute Ribi adjuvant (Ribi Immunochem, Hamilton, MT) and each mouse was injected with 200 µl (2.4 mg protein) of the emulsified preparation. Negative control group received 200 µl of PBS emulsified with the Ribi adjuvant.

EXAMPLE 6

Determination of antileukotoxin antibodies induced by immunization

Blood for serum separation was collected from the right saphenous vein of each mouse on days 0, 21 and 42, and directly from the heart after euthanasia. Antileukotoxin antibody titers were assayed by an indirect ELISA as described previously with slight modifications. Briefly, 96-well microtiter plates (Falcon Probind assay plates, Beckton Dickinson Labware, Lincoln Park, New Jersey) were coated with 50 µl (2 µg/ml) per well of affinity purified native leukotoxin at 37°C for 2 hours. The wells were blocked with 3% bovine serum albumin (Sigma Chemical Company, St. Louis, Missouri) in PBS at 37°C for 2 hours. Fifty µl of a 1 in 25 dilution of serum samples in PBS-T (0.05% Tween 20 in PBS) were added in duplicate and the plates were incubated at 37 °C for 1 hour. Following 6 washes with PBS-T, 100 µl of biotinylated goat anti-mouse immunoglobulin (Accurate Chemicals and Scientific Corp., Westbury, New York) was added to each well and incubated at 37°C for 1 hour. The plates were washed 6 times with PBS-T and 50 µl of streptavidin conjugated with horseradish peroxidase was added to each well, and incubated at 37°C for 1 hour. After washing the wells 6 times with PBS-T, 100µl of ABTS substrate (2,2'-azino-di-[3-ethyl-benzthiazoline-6-sulfonic acid]; Sigma) and H_2O_2 in phosphate-citrate buffer (pH 4.0) was added to each well, and the plates were incubated for 30 min, or until color development, at room temperature. The absorbance was measured colorimetrically at 410 nm in a 96-well plate reader (Molecular Devices, California).

EXAMPLE 7

Experimental challenge with Fusobacterium necrophorum

Fusobacterium necrophorum subsp.*necrophorum*, strain A25 was grown to an OD_{660} of 0.7 in PRAS-BHI broth and 0.4 ml of this late-log-phase culture was injected

intraperitoneally in mice. The inoculum had a bacterial concentration of $4.7x10^8$ CFU/ml as determined by spread-plating on blood agar plates (Remel, Lenexa, Kansas) incubated in an anaerobic glove box (Forma Scientific, Marietta, Ohio). Mice were observed for 4 days post-challenge to record clinical signs and mortality. Mice that survived for 4 days post-challenge were euthanized, necropsied and examined for the presence of abscesses in liver and other internal organs.

EXAMPLE 8

Enumeration of Fusobacterium necrophorum load in the liver

Livers from mice were collected at necropsy, weighed and homogenized in a tissue homogenizer for 1 min in PRAS-BHI broth. A 10-fold dilution of the homogenate was taken inside an anaerobic Glove box for further processing. Two hundred µl of modified lactate medium was dispensed into each well of the 96-well tissue culture plate (Falcon, Beckton Dickinson Labware, Lincoln Park, New Jersey). Fifty µl of 1 in 10 dilution of homogenated liver was transferred to the wells on the first lane (8 wells) and serially diluted (five-fold) up to the eleventh well. The wells in the 12th lane were negative controls. The plates were incubated in a Glove box at 39°C for 48 hours. Kovac's reagent (20 µls each) was added to each well to detect indole production, presumptive of *F. necrophorum*. The bacterial load of *F. necrophorum* in liver was enumerated by most probable number (MPN) analysis (Rowe, R., Todd, R., and Waide, J. 1977. Microtechnique for most-probable-number analysis. Appl. Environ. Microbiol. 33:675-680.). Homogenized liver tissue samples were also streaked on blood agar plates and colonies identified using Rapid ANAII system (Innovative Diagnostic Systems, Norcross, Georgia).

EXAMPLE 9

Statistical Analyses

Serum ELISA measurements (absorbance values per ml of serum) were analyzed using Proc Mixed procedure of SAS (SAS systems, Cary, North Carolina). The weights of liver and bacterial counts, log-transformed, were analyzed using PROC GLM program of SAS. P-values less than 0.01 were considered significant.

Results

Cloning and expression of leukotoxin gene truncations

In-frame cloning of the PCR amplified truncations of the leukotoxin gene (*lkt*A) in plasmid pQE 30 was carried out as described above by incorporating restriction sites for *Sac*I and *Xma*I in the forward and reverse primers respectively. Inducing the clones carrying various truncations did not produce inclusion bodies in the *E. coli* host cells. However, purification of the expressed polypeptides under native conditions was unsuccessful. Therefore, polypeptides were purified using nickel affinity columns after denaturation with guanidium isothiocyanate. The denatured truncated polypeptides, after dialysis against PBS, lacked toxicity to PMNs.

Antileukotoxin antibody titers in rabbits.

The anti-leukotoxin antibody titers in rabbits injected with truncated polypeptides are shown below in Table 4. Antisera raised against truncated leukotoxin polypeptides, BSBSE and GAS, neutralized the toxicity of affinity purified native leukotoxin against bovine peripheral PMNs. The neutralizing activities for polyclonal antisera raised against BSBSE and GAS were similar as evident from their identical neutralization ratios (0.146).

Table 4. Anti leukotoxin antibody titers in rabbits injected with truncated leukotoxin proteins

Truncated proteins	Size (in daltons)	LISA titer on day 21	LISA Titer on day 42 (b)	Neutralization titer on day 42 (a)	Neutralization ratio (a/b)
BSBSE	40810	1250	10000	1460	0.146
SX	97453	1000	8750	0	0
GAS	71949	1150	8750	1280	0.146
SH	63457	1000	10000	0	0
FINAL	80590	875	9750	0	0

Anti-leukotoxin antibody response in mice

The mean absorbances per ml of serum, determined by ELISA, from mice vaccinated with various leukotoxin polypeptides are shown in Table 5.

Table 5. Anti-leukotoxin antibody response in mice injected with various leukotoxin preparations.

Vaccine Preparations	D 0	D 21	D 42	D 46 (post-mortem)
PBS	63.6[a]	65.3[a]	66.9[a]	126.3[d]
BSBSE	52.9[a]	90.2[b]	179.4[c]*	129.1[d]
SX	54.1[a]	77.6[ab]	186.4[c]*	144.5[d]
GAS	61.0[a]	77.6[ab]	97.1[bc]*	109.6[cd]
SH	60.95[a]	101[b]*	163.8[c]*	130.0[d]
FINAL	63.9[a]	66.2[ab]	95.7[bc]*	121.7[cd]
BSBSE+GAS	79.7[a]	82.5[a]	161.1[c]*	172.7[cd]*
ALL FIVE	66.1[a]	98.9[b]*	189[c]*	219[d]*
Native Leukotoxin	59.6[a]	101.3[b]*	235.5[c]*	205.2[d]*
Culture Supernatant	76.4[a]	105.7[b]*	205.4[c]*	230.1[cd]*

Numbers with same superscripts were not significantly different from the ELISA values from mice belonging to same group at a different sampling period.
*Significantly different from negative control (PBS).

On day 21, mice vaccinated with affinity purified native leukotoxin, truncations BSBSE or SH, mixture of all five, or culture supernatant had higher antileukotoxin antibody levels compared to day 0. Serum collected on day 21 from groups vaccinated with truncated polypeptide SH, mixture of five truncations, native affinity purified leukotoxin or culture supernatant, had significantly higher anti-leukotoxin antibody levels compared to the control (PBS) group ($p<0.01$). There was no significant rise in the antibody levels on day 21 among mice vaccinated with truncated polypeptides SX, GAS, FINAL, a combination of BSBSE and GAS or PBS. Mice belonging to group that was vaccinated with culture supernatant, had significantly higher ($P < 0.01$) antibody titers to leukotoxin than mice in other groups.

On day 42, there was a significant increase in antibody response compared to day 21 among mice vaccinated with all leukotoxin preparations except GAS ($P< 0.01$). Anti-leukotoxin antibody levels in serum from mice vaccinated with different leukotoxin

polypeptides (including GAS) were significantly higher compared to the control. The antibody response to a mixture of BSBSE+GAS was similar to BSBSE alone but higher than GAS polypeptide. The antibody response to mixture of all five was similar to BSBSE, SX, SH but higher than GAS or FINAL polypeptides. Mice vaccinated with affinity purified native leukotoxin had the highest anti-leukotoxin antibody levels on day 42, followed by those vaccinated with the culture supernatant and a mixture of all five overlapping truncations. The truncated polypeptide GAS failed to raise anti-leukotoxin antibody levels significantly after the second vaccination compared to the day 21.

On day 46, 4 days after challenge with *F. necrophorum* (post-mortem), serum samples from mice vaccinated with leukotoxin polypeptides, BSBSE, SX, and SH, and affinity purified native leukotoxin had lower anti-leukotoxin antibody titers compared to day 42. Anti-leukotoxin antibody levels in mice vaccinated with GAS, FINAL, mixture of truncated polypeptides or culture supernatant had higher antibody levels compared to day 42. Also, anti-leukotoxin antibody levels in mice in the control group (vaccinated with PBS) on day 46 showed a significant increase than serum collected before challenge (day 42). However, antibody levels in mice injected with BSBSE+GAS, mixture of all five, native leukotoxin and culture supernatant were higher than the control group.

Experimental infection

Following the challenge with *F. necrophorum,* mice in all groups exhibited acute shock within 24 hours perhaps induced by LPS. Mice in the control or in the group vaccinated with inactivated culture supernatant seemed to be affected most. The mice were listless, recumbent and did not seem to consume food or water. Mice vaccinated with various leukotoxin preparations recovered after 2 days post-challenge. Mice in the control group did not recover completely from the symptoms of shock even by day 4 after challenge. Two mice in the control group and one mouse in the group vaccinated with GAS polypeptide died about 36 hours after challenge. Pure cultures of *F. necrophorum* subsp. *necrophorum* were isolated from the heart blood of all three mice.

Hepatic pathology

Mice were euthanized 4 days after challenge and the internal organs were examined for abscesses. None of the mice vaccinated with leukotoxin truncation SH had any liver abscesses (Table 6).

Table 6. Mortality, liver abscess formation, weight of liver and bacterial load in liver in mice vaccinated with leukotoxin preparations after experimental challenge with *Fusobacterium necrophorum*.

Leukotoxin preparations	Number of dead mice	No. of mice with liver abscess (%)	Average weight of liver (g)	MPN counts in the liver
Control (PBS)	2/10	0/8 (0)[a]	1.86	5.3×10^6
BSBSE	0/10	1/10 (10)	1.29*	1.2×10^3 *
SX	0/10	5/10 (50)	1.39*	8.2×10^5 *
GAS	1/10	3/9 (33)	1.32*	1.5×10^6
SH	0/10	0/10 (0)	1.20*	5.3×10^2*
FINAL	0/10	3/10 (30)	1.44*	6.8×10^5 *
BSBSE+GAS	0/10	3/10 (30)	1.27*	1.4×10^5 *
ALL FIVE	0/10	3/10 (30)	1.33*	5.5×10^5 *
Native leukotoxin	0/10	3/10 (30)	1.31*	5.9×10^4 *
Culture supernatant	0/10	1/10 (10)	1.51*	1.6×10^4 *

*Differs from the control group ($P<0.01$)
[a]Livers lacked abscesses, but were highly congested and icteric.

The eight mice that survived in the control group had highly congested and icteric livers, but had no abscesses. Thirty percent of mice vaccinated with affinity purified native leukotoxin, truncations GAS or FINAL, or mixtures (BSBSE and GAS, or all five truncations) had liver abscesses. Five out of ten mice vaccinated with leukotoxin truncated polypeptide SX developed liver abscesses. However, in the groups vaccinated with the truncated leukotoxin polypeptide BSBSE or inactivated culture supernatant, only one out of 10 had liver abscesses.

The mean weight of livers from the control group was significantly higher than mean weights of livers from other groups. Livers from the group that received inactivated culture

supernatant had the next biggest liver size. This correlated with the clinical signs of acute shock displayed by these two groups.

Enumeration of F. necrophorum in liver tissue

Fusobacterium necrophorum subsp. *necrophorum* was isolated from homogenized liver tissue and abscesses from all mice. The counts of*F. necrophorum* from livers of mice injected with any leukotoxin preparation were lower ($p < 0.01$) than the control (Table 6). Livers from mice vaccinated with leukotoxin truncations BSBSE or SH showed significantly lower bacterial counts ($p < 0.01$) than mice vaccinated with other preparations. Among leukotoxin truncations, SX showed least protection followed by FINAL and GAS polypeptides as evidenced by the bacterial counts in the livers of mice vaccinated with these polypeptides. Bacterial counts were considerably lower among groups vaccinated with mixtures of leukotoxin truncations (BSBSE and GAS or all five truncations), or affinity purified native leukotoxin as compared to the control group but higher than SH, BSBSE or inactivated culture supernatant (Table 6).

The five overlapping truncated leukotoxin polypeptides created allowed expression of the entire leukotoxin gene without toxicity to the *E. coli* host cells. Primers for the amplification of various truncated leukotoxin gene products were designed in such a way that the expressed polypeptides were not toxic to *E. coli* host cells, but were big enough (at least 30 kDa) to be a good immunogen. The nickel affinity column purified polypeptides were tested for purity in terms of contaminating proteins or lipopolysaccharides by silver-staining the SDS-PAGE separated proteins. Because all truncated polypeptides were purified under denaturing conditions, they were not toxic as determined by the MTT assays. *Fusobacterium necrophorum* culture supernatant and affinity purified native leukotoxin were inactivated with 0.3% formalin before injection, thus were nontoxic.

Neutralization of toxicity of *F. necrophorum* leukotoxin against bovine peripheral PMNs by antiserum raised against BSBSE and GAS polypeptides suggested that biologically important domains, such as those responsible for toxicity or host cell receptor binding was located in these regions. Therefore, a mixture of these two polypeptides (BSBSE+GAS) was also used in a vaccine preparation in our challenge experiments with mice.

The significantly higher antibody levels noticed among groups vaccinated with preparations containing full-length leukotoxin proteins (native affinity purified leukotoxin, culture supernatant, or a mixture of recombinant leukotoxin polypeptides containing all five truncations) may be due to determinant spreading, or due to augmentation of anti-leukotoxin antibody response by the presence of multiple immunodominant epitopes on the leukotoxin protein. Truncated leukotoxin GAS produced a low antibody response. The high hydrophobicity of this polypeptide may be the reason for its reduced immunogenicity. Also, the wells in the ELISA plates were coated with native immunoaffinity purified leukotoxin, and the domains represented by the GAS polypeptide could possibly be hidden and not exposed for the antibodies against GAS polypeptide to bind.

Decrease in anti-leukotoxin antibody levels among various groups of mice on day 46 (4 days after experimental challenge with *F. necrophorum*) suggested neutralizing effect and clearance of toxin secreted by *F. necrophorum* used for experimental challenge by these antibodies. Pure cultures of *F. necrophorum* subsp. *necrophorum* were isolated from the heart blood of the three mice (two from negative control group and one from group injected with GAS polypeptide) that died on day 2 after challenge, suggesting that death was due to septicemia induced by *F. necrophorum.* The hepatic tissue from the negative control group showed inflammation, congestion and icterus characteristic of an acute phase response, but showed no abscesses.

Multiple responses including mortality, clinical signs, weights of liver, presence of abscesses, and the bacterial load in liver were considered to evaluate the effectiveness of various vaccine preparations in providing immunity and protection against experimental challenge with *F. necrophorum*. Leukotoxin truncation SH was a very effective immunogen as evidenced by a rise in anti-leukotoxin antibody levels in serum samples on day 21 or 42. Also, there were no mortality, hepatic inflammation or abscesses in mice vaccinated with this polypeptide after experimental challenge. The mean bacterial load in the livers of mice from this group was the lowest (5.3×10^2). Interestingly, leukotoxin truncated polypeptide SH did not induce neutralizing antibodies in rabbits. Production of high-affinity antibodies against certain immunodominant domains that brings about effective opsonization and clearance of leukotoxin in an experimental challenge model may render this truncated polypeptide (SH) a protective antigen.

Vaccination with N-terminal truncation BSBSE or culture supernatant followed by experimental challenge with *F. necrophorum* caused no mortality, but livers were abscessed in 10% of the mice. Mice vaccinated with BSBSE, however, had less clinical signs of LPS induced shock after vaccinations or challenge, lower liver weights and lower hepatic-bacterial counts compared to mice vaccinated with inactivated culture supernatant.

Native leukotoxin purified by immunoaffinity columns from *F. necrophorum* culture supernatant was the fourth best vaccine preparation (behind SH, BSBSE, and culture supernatant) in terms of serum antibody levels, protection against formation of liver abscess (30%), and number of bacteria in the liver tissue. The vaccine consisting of a mixture of all five recombinant truncated leukotoxin polypeptides also protected 70% of mice from abscess formation and the bacterial counts in their hepatic tissue were not significantly different from mice that were vaccinated with native leukotoxin.

Truncated polypeptide GAS, although it invoked neutralizing antibodies in rabbits, was a poorer immunogen and protected 67% of the mice in its group from formation of liver abscesses but one of the ten mice in this group died after challenge. As mentioned above, this region could contain domain(s) of toxicological importance such as, target cell binding, biological activities. However, multiple host-factors such as, availability of specific lymphocyte sub-population for clonal selection, type of helper T-cells stimulated, ability to invoke antibodies capable of opsonization, decide if an antibody response to a particular protein is protective in the species of animal tested.

The truncated leukotoxin polypeptide SX provided least protection from liver abscess formation. The number of bacteria in the hepatic tissue of mice vaccinated with GAS or SX were significantly higher ($P < 0.01$) than in livers of mice vaccinated with SH, BSBSE, culture supernatant or full-length native or recombinant leukotoxin (mixture of five truncations), but was lower than the mice in the negative control group. A mixture of BSBSE and GAS or the FINAL polypeptides provided only a mediocre protection against experimental challenge. Polyclonal antisera raised in rabbits against BSBSE or GAS neutralized the activity of native leukotoxin against PMNs used as target cells and were thus chosen to be used in combination.

Recombinant truncated leukotoxin polypeptides SH and BSBSE provided significant protection in mice when used as a vaccine individually. Dilution of immunodominant and

protective epitopes present within these regions by including other truncated polypeptides as seen in vaccine preparations containing affinity purified leukotoxin or combinations of truncated leukotoxin polypeptides possibly caused a decrease in overall protection. Further studies to test the effectiveness of leukotoxin truncations BSBSE and SH individually or in combination providing protection against natural or experimental infections with *F. necrophorum* infections need to be carried out. This study provided further credence to the importance of leukotoxin as the major virulence factor of *F. necrophorum* and the protein carries a domain (s) or epitope (s) that induces protective immunity against experimental infection. The vaccine that produced best antileukotoxin titer did not always afford good protection against experimental infection. Therefore, certain epitopes may be more important in conferring protective immunity to infection. The results of this study suggest that some of these important epitopes reside on the BSBSE and SH polypeptides.

Discussion

Fusobacterium necrophorum subsp. *necrophorum* is isolated more often than subsp. *funduliforme* from necrotic abscesses. The strains of subsp. *necrophorum* produces the high molecular weight leukotoxin in greater quantities than strains of subsp. *funduliforme*. In this study, we have cloned the leukotoxin gene from the highly virulent *F. necrophorum* subsp. *necrophorum* strain A25. The evidence that the *lkt*A determinant encodes the leukotoxin is as follows: (1) the ORF encodes a 336 kDa protein, a size consistent with previous studies of the toxin; (2) the protein encoded by the recombinant *lkt*A determinant is recognized by both polyclonal and monoclonal antibodies raised against purified leukotoxin from *F. necrophorum*; (3) antisera raised against polypeptides from the cloned *lkt*A determinant recognized the native toxin in western blots; (4) antisera raised against two of the truncated polypeptides neutralized the toxic activity of the leukotoxin; and (5) the recombinant protein expressed in *E. coli* is relatively more toxic to bovine neutrophils as compared to bovine lymphocytes. These differing degrees of toxicity toward neutrophils relative to lymphocytes is also observed with leukotoxin that was affinity-purified from *F. necrophorum* culture supernatants.

The leukotoxin ORF is 9,726 base pairs long encoding a 3,241 amino acid protein with an overall molecular mass of 335,956 daltons. The DNA and deduced amino acid

sequences were compared with sequences in Genbank but no significant (greater than 25% identity) similarities were found with other bacterial toxins. For example, the closest identity was found with *Hmw*A from *Haemophilus influenzae* (22% or 356 out of 1,625 residues). Other similar homologies were found in *Srp*A from *Streptococcus cristatus* (17% or 388 out of 2,239 residues), *Omp*A from *Ricketsia australis* (21% or 321 out of 1,489 residues) and the 190 kDa surface antigen of *Rickettsia ricketsii* (21% or 379 out of 1,770 residues). Other Thus, the *F. necrophorum* leukotoxin appears to be distinct from all known leukotoxins and RTX-type toxins. When the deduced amino acid sequence of the *lkt*A region was subjected to the Kyte-Doolittle hydropathy analysis (Fig. 3), 14 sites of sufficient length and hydrophobic character to be potential membrane spanning regions, were found. Upstream to the leukotoxin ORF is an open reading frame of at least 1.4 kb in length, which is in the same orientation. It encodes a protein that has some sequence identity to the heme-hemopexin utilization protein (UxuB) of *Haemophilus infuenzae*.

Additionally, the protein is larger than any bacterial exotoxins identified to date and shows no sequence similarity to other known leukotoxins. Thus, this protein may represent a new class of bacterial leukotoxins. The protein is unusual in that it is devoid of cysteine. This is not a characteristic of proteins from anaerobes, as evidenced by the normal content of cysteine residues in the clostridial toxins including *Clostridium botulinum* neurotoxin, *Cl. difficile* cytotoxin B, *Cl. septicum* alpha-toxin, and *Cl. tetani* tetanus toxin (Genbank accession numbers AB037166, AB217292, D17668, and X06214, respectively). The leukotoxin protein has a sequence at its N-terminus that has the properties of a signal sequence. This may indicate that the protein is exported across the cytoplasmic membrane in *F. necrophorum* in a Sec pathway-dependent manner.

The DNA sequences flanking *lktA* suggests that this toxin gene may be part of a multigene operon with at least one ORF upstream and another downstream of this gene. The activity of the LktA protein expressed in *E. coli* indicates that the other proteins encoded in the putative leukotoxin operon are not required to produce a biologically active toxin. Their role may be in secretion of the toxin across the cytoplasmic and outer membranes of *F. necrophorum* into the culture fluid.

If the *lktA* determinant is part of an operon, it would be greater than 12 kb in length. A dilemma with such a large operon might be to efficiently translate the messenger RNA

species without premature dissociation of ribosome from the message. A peculiarity in the cloned region is an abundance of potential ribosome binding site sequences. Within the cloned region, there are 26 occurrences of GGAGG, which is a perfect match to the sequence at the 3' end of the 16S rRNA. The complementary sequence, CCTCC, which has the same G+C content but does not act as a ribosome binding site, is present only two times in the sequence. The abundance of the GGAGG sequence could provide translation reinforcement sequences to help ensure that a ribosome remains associated with the message and completes the translation of the ORFs. The abundance of the putative RBS sequence (GGAGG) is due to the presence of di-glycine repeats in the amino acid sequence. The GGA glycine codon occurs 263 times in the leukotoxin ORF and 24 of the 26 occurrences of GGAGG in the 11,130 bp sequenced to date correspond to tandem repeats of this codon. This feature of the amino acid sequence in the protein may provide the additional benefit of enabling more efficient translation of the message.

Expressing the 3.5 kb sequence from the 5' end of *lkt*A caused immediate cessation of growth and lysis of *E. coli* carrying this recombinant expression vector. Creation of overlapping truncations allowed the expression of the entire leukotoxin gene without significant toxicity to the *E. coli* host cells. Polyclonal antileukotoxin antiserum reacted strongly to three truncated polypeptides (BSBSE, SX and FINAL) and more weakly to the other two truncated polypeptides (GAS and SH) in western blot analysis. This low reactivity was not due to poor immunogenicity of these relatively hydrophobic polypeptides, because both polypeptides (GAS and SH), produced high antibody titers in rabbits. Thus, it may been due to the tertiary folding pattern of leukotoxin under native conditions. The toxin being a secreted protein, would have its hydrophobic domains internalized when the protein was properly folded. The epitopes corresponding to these domains may not be as accessible to the immune system. Antibodies against these epitopes would thus be underrepresented when the whole un-denatured toxin is used as the immunogen. Interestingly, antibodies to one of these polypeptides, GAS, was neutralizing. Thus at least some of the critical epitopes are available in the active toxin.

The intact leukotoxin gene was introduced into *E. coli* under the control of the *lac* promoter. Inducible expression of full-length leukotoxin protein was achieved without any recognizable toxicity to *E. coli* host cells. Expression of the full-length leukotoxin instead

of truncated polypeptides may allow correct folding of the toxin. This would result in internalization of the hydrophobic domains with a corresponding reduction of toxicity in *E. coli* host cells. Both polyclonal and monoclonal antibodies against native leukotoxin recognized a protein species with a size consistent with that of the intact leukotoxin in western blot analysis of cell lysates of *E. coli* harboring pSN2000. Antibodies raised against all five truncated leukotoxin polypeptides, but not the upstream polypeptide, recognized full-length recombinant leukotoxin as well.

In order to determine the prevalence and heterogeneity of leukotoxin gene in this species, 15 *F. necrophorum* strains belonging to subsp. *necrophorum* and subsp. *funduliforme* isolated from liver abscesses (opportunistic pathogen) or rumen contents (normal inhabitant) were screened for *lkt*A by Southern blotting. Strains belonging to *F. necrophorum* subsp. *necrophorum,* irrespective of its location of isolation (liver abscess or ruminal contents) had similar hybridizing patterns. Similarly, all strains of *F. necrophorum* subsp. *funduliforme*, irrespective of the site from which it was isolated had identical hybridization patterns, but which differed from the subspecies *necrophorum* pattern. The difference in Southern blot hybridization patterns suggest that the disparity in levels of leukotoxin produced between the two subspecies may be due to differences in genetic organization of the leukotoxin locus.